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# Hyperhomocysteinaemia is not associated with isolated crural arterial occlusive disease: The Hoorn Study

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**Abstract.** Hoogeveen EK, Kostense PJ, Jakobs C, Rauwerda JA, Dekker JM, Nijpels G, Bouter LM, Heine RJ, Stehouwer CDA (University Hospital Vrije Universiteit, Amsterdam). Hyperhomocysteinaemia is not associated with isolated crural arterial occlusive disease: the Hoorn Study. *J Intern Med* 2000; **247**: 442–448.

**Objectives.** Hyperhomocysteinaemia is an independent risk factor for peripheral arterial disease (PAD). The localization of peripheral arterial disease is clinically relevant, because proximal (aortoiliac and femoropopliteal) disease is associated with a particularly poor overall prognosis, whereas isolated distal (i.e. crural) disease is associated with a better overall prognosis. The aim of the study was to investigate whether the strength of the association between hyperhomocysteinaemia and peripheral arterial disease differs according to the localization of the anatomical obstruction.

**Design.** Fasting serum total homocysteine (tHcy) was measured in an age-, sex- and glucose-tolerance stratified random sample ( $n = 631$ ) of a 50- to 75-year-old general Caucasian population. History of a peripheral arterial reconstruction was recorded.

Aortoiliac, femoropopliteal and crural arterial obstructions were registered by means of Doppler flow velocity curves.

**Results.** The median serum tHcy level was  $12.2 \mu\text{mol L}^{-1}$  (interquartile range: 10.0–15.3) in men and  $10.7 \mu\text{mol L}^{-1}$  (interquartile range: 9.0–13.3) in women. The prevalences of aortoiliac, femoropopliteal and crural obstructions were 2.1%, 2.7% and 11.9%, respectively. After adjustment for age, sex, systolic blood pressure, current smoking, serum cholesterol and diabetes mellitus, the odds ratios (95% confidence interval) per  $5 \mu\text{mol L}^{-1}$  tHcy increment were 1.41 (1.05–1.89) for aortoiliac, 1.03 (0.70–1.52) for femoropopliteal and 0.82 (0.59–1.15) for crural obstructions. Finally, diabetes mellitus, HbA<sub>1c</sub> and current smoking were significantly associated with crural and femoropopliteal disease, whereas systolic blood pressure was significantly associated with aortoiliac obstructions.

**Conclusions.** The present study indicates that hyperhomocysteinaemia is associated with aortoiliac but not with isolated crural arterial occlusive disease.

**Keywords:** atherosclerosis, diabetes mellitus, homocysteine, hypertension, peripheral arterial disease.

## Introduction

Peripheral arterial disease (PAD) has two important clinical consequences: it is associated with an increased risk of overall mortality, presumably because it is a marker of generalized atherosclerosis, and it impairs local blood flow, which may result in ischaemic symptoms, such as intermittent claudication and critical limb ischaemia [1–3]. Peripheral arterial disease can occur from the aortoiliac to the crural territories. The localization of peripheral

arterial disease is clinically relevant, because proximal (aortoiliac and femoropopliteal) disease is often accessible to local treatment, but is thought to be associated with a particularly poor overall prognosis, whereas isolated distal (i.e. crural) disease is often difficult to treat locally, but is associated with a better overall prognosis than proximal arterial disease [4]. It is therefore of interest to investigate whether risk factors for proximal versus distal peripheral arterial disease are different. In this regard, it is well-established that diabetes mellitus

is more strongly associated with distal peripheral arterial disease, whereas smoking, hypertension and hypercholesterolaemia are more closely associated with proximal peripheral arterial disease [5–8].

Hyperhomocysteinaemia is a novel risk factor for cardiovascular disease, which is independent of classic risk factors such as smoking, hypercholesterolaemia, diabetes mellitus and hypertension [9–12]. Although the mechanisms by which homocysteine promotes atherothrombosis are unknown, the epidemiologic evidence of the association of hyperhomocysteinaemia with atherothrombotic disease is strong [9, 13]. A high serum total homocysteine (tHcy) concentration can be lowered with folic acid supplementation [14]. Studies which specifically investigated the relationship between hyperhomocysteinaemia and peripheral arterial disease are relatively scarce [15–19]. It is not known whether the strength of the association between hyperhomocysteinaemia and peripheral arterial disease is similar for proximal and distal disease.

In order to further explore this issue, we compared the strength of the association between tHcy and other risk factors on the one hand and the level of peripheral arterial obstruction (i.e. aortoiliacal versus femoropopliteal versus crural), on the other hand in a 50- to 75-year-old general Caucasian population.

## Patients and methods

### *Study design*

The Hoorn Study is a cross-sectional survey of glucose tolerance and other cardiovascular risk factors in a 50- to 75-year-old general Caucasian population conducted from 1989 to 1992. A random sample of all men and women aged 50–75 years was drawn from the municipal population registry office of Hoorn (the Netherlands); 2484 subjects participated (response rate 71%). An extensive peripheral arterial investigation (detailed below) was performed in an age-, sex- and glucose tolerance-stratified random subsample ( $n = 631$ ; response rate 89%) [20]. The Hoorn Study was approved by the Ethical Review Committee of the University Hospital Vrije Universiteit. Informed consent was obtained from all participants.

The peripheral arterial history was obtained by means of a self-administered questionnaire and, if

positive, accepted only when confirmed by written information of the participant's general practitioner. Flow velocity curves were recorded, by one of two experienced vascular technicians, from the femoral, popliteal, posterior tibial and dorsalis pedis arteries by means of a 5 or 8 MHz bi-directional continuous wave Doppler connected to a real-time frequency analyser. Tri- or biphasic curves indicate a normal arterial inflow to that level. Monophasic or absent curves are considered abnormal, signifying the presence of an obstruction of 50% or more, proximal to the examination site [21–23]. An aortoiliac obstruction was defined as an abnormal Doppler flow velocity curve from the femoral, the popliteal and the three crural arteries, or having received a bifurcation prosthesis; a femoropopliteal obstruction as a normal flow velocity curve from the femoral artery in combination with abnormal curves from the popliteal artery and the crural arteries, or having received a femoropopliteal reconstruction; and a crural obstruction as normal curves from the femoral and the popliteal arteries in combination with an abnormal curve from one or more of the crural arteries. The absence of, or presence of a monophasic Doppler flow velocity curve only from the peroneal artery in combination with normal curves from the other two crural arteries and an ankle brachial pressure index (ABPI)  $>0.9$  in the same limb was considered a technical failure ( $n = 18$ ). The ABPI, an estimate of the overall severity of occlusive disease, was obtained by means of Doppler-assisted systolic blood pressure measurements taken from the brachial and the three crural arteries on both sides as previously described in more detail [20].

### *Measurement of serum total homocysteine*

Fasting blood samples were centrifuged within 1 hour following collection. Serum was stored at  $-20^{\circ}\text{C}$  for 4–6 years. There is good evidence that serum tHcy levels in frozen samples are stable for 10 years or more [24]. Serum total (free plus protein bound) homocysteine level was measured by using tri-*n*-butylphosphine as the reducing agent and ammonium 7-fluorobenzo-2-oxa-1,3-diazole-4-sulphonate as the thiol-specific fluorochromophore, followed by high performance liquid chromatography with fluorescence detection [25]. The intra- and interassay coefficients were 2.1% and 5.1%.

### *Other cardiovascular risk factors*

We measured fasting serum total cholesterol, HDL cholesterol and triglycerides by enzymatic techniques (Boehringer-Mannheim, Mannheim, Germany). The Friedewald formula was used to calculate the low density lipoprotein (LDL) cholesterol concentration, except in subjects with serum triglyceride levels greater than  $8.0 \text{ mmol L}^{-1}$  ( $n = 3$ ) [26]. Blood pressure was measured on the right arm of seated subjects, after at least 5 min of rest, using a random zero sphygmomanometer (Hawksley-Gelman Ltd, Lancing, Sussex, UK). The average of duplicate measurements on two occasions was used for analysis. Hypertension was defined as a blood pressure  $\geq 160 \text{ mmHg}$  systolic and/or  $\geq 95 \text{ mmHg}$  diastolic and/or the current use of antihypertensive medication. Normal glucose tolerance, impaired glucose tolerance and diabetes mellitus were defined according to the WHO criteria (1985) [27] applied to the mean of two oral glucose tolerance tests, except in patients with drug-treated diabetes mellitus, as previously described in detail [20]. Glycated haemoglobin ( $\text{HbA}_{1c}$ ) was determined by an ion-exchange high-performance liquid chromatography, using a Modular Diabetes Monitoring System (Bio-Rad, Veenendaal, the Netherlands). Immunospecific insulin was measured in serum by a double-antibody radioimmunoassay (lot SP21, Linco Research, St Louis, USA). The interassay coefficient of variation was 6%. The lower limit of sensitivity was  $12 \text{ pmol L}^{-1}$ . Subjects were classified as either nonsmoker or current smoker. Body mass index (BMI) was calculated as weight divided by height squared ( $\text{kg m}^{-2}$ ). Waist and hip circumferences were measured and the waist-hip ratio (WHR) was calculated as described elsewhere [20]. Central adiposity was defined as a WHR  $> 1.00$  in men and  $> 0.90$  in women. All laboratory and vascular measurements were carried out by technicians unaware of the subjects' history of peripheral arterial disease and glucose tolerance status.

### *Statistical analysis*

Variables are presented as mean  $\pm$  standard deviation (SD), number (percentage of the total) or, in case of skewed distribution, median and interquartile range). The most proximal obstruction of either limb was used for statistical analysis. We performed

logistic regression analyses to study the associations of serum tHcy and of other cardiovascular risk factors with each level of peripheral arterial disease. As the dependent variable we took aortoiliac, femoropopliteal or crural obstruction and contrasted each group with subjects without any peripheral arterial disease. We chose this procedure because, in subjects with aortoiliac or femoropopliteal disease, more distal (i.e. femoropopliteal or crural, respectively) disease cannot be excluded. For tHcy, we calculated odds ratios (ORs) and 95% confidence intervals (CIs) per  $5 \text{ } \mu\text{mol L}^{-1}$  (about 1 SD) increment of serum tHcy. We used multiple logistic regression analysis, after adjustment for age and sex, to investigate the associations between level of peripheral arterial disease and systolic blood pressure, serum total cholesterol, current smoking, diabetes mellitus and tHcy. We also tested models which included triglycerides, HDL and LDL cholesterol, glucose,  $\text{HbA}_{1c}$ , insulin, BMI and/or WHR. The small number of cases with aortoiliac or femoropopliteal obstruction did not allow for extensive multivariate adjustment, nor for analyses of hypertension as the dichotomous variable. We repeated all analyses without taking peripheral arterial reconstructions into account. All reported *P*-values are two-tailed. All analyses were performed with SPSS for Windows 7.5.2.

## **Results**

Table 1 shows the characteristics of the study population. Ten subjects had previously had a peripheral arterial reconstruction: three of these had had an aortoiliac bifurcation prosthesis and seven a femoropopliteal bypass. Two subjects had previously undergone a limb amputation, so data apply to only one leg. One of these subjects had a traumatic limb amputation, which was not considered as peripheral arterial disease. Table 2 shows the prevalences of peripheral arterial disease according to localization of it. After exclusion of subjects who had undergone reconstructive surgery, an ABPI  $< 0.5$  (a proxy measure of multilevel disease [28]) was present in 50% (five out of 10) of the subjects with an aortoiliac obstruction and 28.6% (four out of 14) of the subjects with a femoropopliteal obstruction. The small number of subjects did not allow further analysis of the determinants of proximal peripheral arterial disease as defined here,

**Table 1** Characteristics of the study population

<i>n</i>	631
Men (%)	48
Age (years)	64.3 (7.2)
Body mass index (kg m <sup>-2</sup> )	27.3 (4.0)
Waist-hip ratio	0.92 (0.09)
Current smoker (%)	29
Systolic blood pressure (mmHg)	139 (19)
Diastolic blood pressure (mmHg)	83 (10)
Hypertension (%)	39.1
Impaired glucose tolerance (%)	26.9
Diabetes mellitus (%)	27.4
HbA <sub>1c</sub> (% of haemoglobin)	5.9 (1.3)
Fasting insulin (pmol L <sup>-1</sup> )	84 (63–119)*
Total cholesterol (mmol L <sup>-1</sup> )	6.6 (1.2)
HDL cholesterol (mmol L <sup>-1</sup> )	1.3 (0.4)
LDL cholesterol (mmol L <sup>-1</sup> )	4.5 (1.1)
Triglycerides (mmol L <sup>-1</sup> )	1.6 (1.1–2.2)*
Total homocysteine (μmol L <sup>-1</sup> )	11.4 (9.3–14.1)*

Data are presented as mean (SD). \*Median (interquartile range).

i.e. without or with multilevel disease, versus the determinants of isolated proximal peripheral arterial disease, i.e. proximal peripheral arterial disease without multilevel disease.

The median serum tHcy level was 12.2 μmol L<sup>-1</sup> (interquartile range: 10.0–15.3) in men and 10.7 μmol L<sup>-1</sup> (interquartile range: 9.0–13.3) in women. We found a positive association between the serum tHcy level and more proximally located lower limb arterial obstruction (Table 2). The odd's ratios did not change materially when subjects with a history of vascular reconstruction were excluded (data not shown).

After adjustment for age and sex, we found that diabetes mellitus, HbA<sub>1c</sub> and current smoking were significantly associated with crural and femoropopliteal obstruction, whereas systolic blood pressure

was significantly associated with aortoiliac obstruction (Table 3). Additional adjustment for use of antihypertensives (yes/no) did not materially change the association between blood pressure and aortoiliac obstruction (odd's ratio 1.26; 1.10–1.46; 95% CI). Total cholesterol was more strongly associated with aortoiliac than distal peripheral obstruction (Table 3), although the association with aortoiliac obstruction was not statistically significant (*P* = 0.3). After adjustment for HbA<sub>1c</sub>, the strength of the association between diabetes and aortoiliac disease was not attenuated, whereas that with femoropopliteal obstruction was. Impaired glucose tolerance was not significantly associated with any obstruction (data not shown). We therefore pooled subjects with normal and impaired glucose tolerance in all analyses. HDL cholesterol, triglycerides, fasting insulin, BMI and WHR were not significantly associated with a specific level of peripheral arterial disease and adjusting for each of these variables did not materially affect the association between diabetes and crural, femoropopliteal or aortoiliac obstruction (data not shown).

We considered subjects with a monophasic Doppler flow velocity curve from only the peroneal artery and an ABPI > 0.9 in the same limb not to have crural disease (*n* = 18; see methods). Categorization of these subjects as having crural disease did not materially affect the results (data not shown).

Finally, the number of premenopausal women (*n* = 13; defined as those women who had menstruated within the last year) was too small to allow for a subanalysis of the association between tHcy and localization of peripheral arterial disease before and after menopause. Exclusion of premenopausal women did not materially affect the results (data not shown).

**Table 2** Odds ratios for proximal and distal peripheral arterial disease per 5 μmol L<sup>-1</sup> increase of serum total homocysteine

Obstruction	Cases	Prevalence (%) (95% CI)	Crude OR (95% CI)	Age- & sex-adjusted OR (95% CI)	Multivariate <sup>a</sup> adjusted OR (95% CI)
Any	105	16.6 (13.7–19.5)	1.08 (0.92–1.27)	1.04 (0.87–1.24)	1.03 (0.85–1.23)
Aortoiliac <sup>b</sup>	13	2.1 (1.1–3.5)	1.37** (1.11–1.68)	1.36** (1.11–1.68)	1.41* (1.05–1.89)
Femoropopliteal <sup>c</sup>	17	2.7 (1.6–4.3)	1.11 (0.82–1.50)	1.06 (0.71–1.57)	1.03 (0.70–1.52)
Crural	75	11.9 (9.4–14.4)	0.87 (0.67–1.14)	0.81 (0.59–1.11)	0.82 (0.59–1.15)

<sup>a</sup>Adjusted for age, sex, diabetes mellitus (yes/no), systolic blood pressure, current smoking (yes/no) and serum cholesterol. <sup>b</sup>Aortoiliac obstruction (*n* = 10) and/or bifurcation prosthesis (*n* = 3). <sup>c</sup>Femoropopliteal obstruction (*n* = 14) and/or femoropopliteal reconstruction (*n* = 7). \**P* < 0.05; \*\**P* < 0.01. OR: odds ratio, CI: confidence interval. Note: each level of peripheral arterial obstruction was contrasted with subjects without any obstruction.

**Table 3** Odds ratios (95% confidence intervals) for aortoiliac, femoropopliteal and crural arterial obstructions of selected cardiovascular risk factors

	Aortoiliac <sup>b</sup> obstruction ( <i>n</i> = 13) age- & sex-adjusted OR (95% CI)	Femoropopliteal <sup>c</sup> obstruction ( <i>n</i> = 17) age- & sex-adjusted OR (95% CI)	crural obstruction ( <i>n</i> = 75) age- & sex-adjusted OR (95% CI)	crural obstruction ( <i>n</i> = 75) multivariate adjusted <sup>a</sup> OR (95% CI)
Diabetes mellitus (yes/no)	2.77 (0.90–8.51)	4.33** (1.58–11.87)	1.98** (1.19–3.28)	1.11 (0.54–2.25)
HbA <sub>1c</sub> (per percentage)	1.22 (0.80–1.84)	1.65*** (1.26–2.16)	1.42*** (1.21–1.67)	1.42*** (1.14–1.76)
Current smoking (yes/no)	1.34 (0.39–4.53)	4.19*** (1.53–11.51)	2.49*** (1.47–4.21)	2.65*** (1.54–4.57)
Total cholesterol (per mmol L <sup>-1</sup> )	1.28 (0.83–1.99)	1.15 (0.77–1.71)	1.00 (0.82–1.23)	0.95 (0.77–1.17)
Systolic blood pressure (per 5 mmHg)	1.29*** (1.13–1.46)	1.09 (0.97–1.24)	0.94 (0.88–1.01)	0.93 (0.87–1.00)

<sup>a</sup>Adjusted for age, sex and diabetes mellitus (yes/no), current smoking (yes/no), and HbA<sub>1c</sub> if this was not the variable under consideration.

<sup>b</sup>Aortoiliac obstruction and/or bifurcation prosthesis. <sup>c</sup>Femoropopliteal obstruction and/or femoropopliteal reconstruction. \**P* < 0.05;

\*\**P* < 0.01; \*\*\**P* < 0.001. OR: odds ratio, CI: confidence interval. Note: after adjustment for age, sex and HbA<sub>1c</sub>, the ORs (95% CIs) for the association between diabetes mellitus and aortoiliac and femoropopliteal obstruction were 2.91 (0.77–11.00) and 2.06 (0.55–7.63).

## Discussion

There are two main findings of this study. Firstly, hyperhomocysteinaemia is more strongly associated with proximal than with distal peripheral arterial disease. Secondly, diabetes, level of glycaemia (estimated from HbA<sub>1c</sub>) and current smoking were associated with crural and femoropopliteal disease, whereas systolic blood pressure was associated with aortoiliac obstructions.

Others reported that hyperhomocysteinaemia is associated with peripheral arterial disease [10, 15–18]. The current study brings the association between hyperhomocysteinaemia and localization of peripheral arterial disease into focus. Although there are no previous studies that have addressed this issue, several observations from studies in which serum total homocysteine (tHcy) was not measured may support this finding. Firstly, premature peripheral atherosclerosis (i.e. disease affecting those under 50 years) is often located in the aortoiliac bed, i.e. proximally [5], and is known to be associated with hyperhomocysteinaemia [15, 16, 18]. Secondly, aortoiliac obstruction without distal occlusions occurs twice as often in (premature) postmenopausal compared with premenopausal women [29, 30], and postmenopausal women have higher serum tHcy levels than premenopausal women [31]. Several pathophysiological mechanisms have been proposed through which hyperhomocysteinaemia may induce atherosclerosis. Hyperhomocysteinaemia may induce dysfunction of the vascular endothelium [32–34], a critical initiating event in the development of atherosclerosis. In addition,

hyperhomocysteinaemia may stimulate proliferation of vascular smooth muscle cells and elastolytic processes in the arterial wall [34–37]. Atherosclerosis occurs at definite sites of predilection within the vascular tree, such as bifurcations, angulations or fixed points. The abdominal aorta is at high risk of atherosclerosis due to the thickness of the avascular zone of the arterial wall, which increases the risk of ischaemia. However, it is not known how hyperhomocysteinaemia affects the mechanisms leading to aortoiliac as opposed to more distal atherosclerosis.

This study confirms that diabetes is associated with distal peripheral arterial disease [4, 8]. After adjustment for HbA<sub>1c</sub> the association between diabetes and crural or femoropopliteal obstruction disappeared. Adjustment for other factors of the insulin resistance syndrome did not affect the associations. This result may indicate that hyperglycaemia itself is more important with regard to the development of crural obstruction than other factors of the insulin resistance syndrome [38]. Finally, the present study also confirms that systolic blood pressure is more closely associated with proximal arterial disease, and, in addition, suggests that systolic blood pressure is probably not a risk factor for crural disease.

We have to consider several limitations of the present study. Firstly, we relied on a noninvasive technique to assess peripheral arterial disease which detects the most proximal obstruction rather than the degree or extent of the obstruction. Although angiography is not suitable for a population-based study, duplex scanning would have provided more precise information on the extent and severity of

obstruction. As a consequence, we could not distinguish whether hyperhomocysteinaemia is especially associated with obstructions confined to the aortoiliac region or with multilevel disease [15]. The small number of subjects with an ABPI < 0.5, a proxy of multilevel disease [28], did not allow further analysis of this issue. Secondly, since the present study is cross-sectional, we cannot rule out that hyperhomocysteinaemia is a consequence of the disease rather than a cause. However, there is increasing evidence that the relation between tHcy and cardiovascular disease is causal [9, 11, 12, 32–37]. Finally, the small number of subjects with a proximal arterial obstruction warrants careful interpretation of the results of the present study.

Proximal aortoiliac disease is associated with a particularly poor overall prognosis. Therefore, the results of the present study may have clinical relevance because hyperhomocysteinaemia can effectively be lowered through an increased intake of B vitamins, particularly folate [14].

In conclusion, hyperhomocysteinaemia and systolic blood pressure are more strongly associated with proximal than with distal peripheral arterial disease, whereas diabetes and level of glycaemia are associated with distal and possibly with proximal peripheral arterial disease.

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